Exhibit 2

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Inheritance and Tissue-Specific Expression of Transgenes in Rabbits and Pigs

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INTRODUCTION: GROWTH HORMONE GENES

Since 1985 when for the first time transgenic livestock were reported (Hammer et al., 1985; Brem et al., 1985) a number of research groups have used this technique, particularly for producing transgenic pigs.

Most experiments in genetic engineering of livestock have been carried out to produce elevated levels of growth hormone for improving weight gain and feed efficiency, as well as to achieve a marked reduction in fat. However, these achievements were offset by a high incidence of lesions of kidney, liver, and heart in mice (Brem et al., 1989) and of gastric ulcers, arthritis, cardiomegaly, dermatitis, and renal disease in pigs (Pursel et al., 1989). Our transgenic pigs with a MT-GHRH (growth hormone releasing hormone) minigene did not show any alterations in production characteristics because of insufficient expression patterns.

Mx INFLUENZA RESISTANCE GENES

Orthomyxovirus infections are common and occasionally cause epidemic diseases not only in man but also, for example, in pigs. Genetic analyses have established an antiviral state against influenza virus in mice carrying the autosomal dominant $Mx1^+$ allele (Lindenmann et al., 1963). The Mx 1 gene in mice has been cloned and functionally characterized (Staeheli et al., 1986). Mx is an interferon-stimulated protein and Mx1 protein is both necessary and sufficient to promote resistance to influenza virus infections (for review, see Müller and Brem, 1991). Mx homologs exist in all examined eukaryotes and may carry out functions that are fundamental to cellular physiology. Our DNA, RNA, and protein studies in IFN-treated porcine peripheral blood lymphocytes have indicated the existence of at least two Mx genes in pigs (Müller et al., 1992a).

In our gene transfer experiments in pigs we have used three different gene constructs consisting of the murine Mx 1 cDNA placed under the transcriptional control of the human metallothionein II_A promoter, the SV 40 early enhancer/promoter, or the murine Mx promoter itself (Weissmann and Noteborn, 1986, pers. communication). The efficiencies of the generation of transgenic pigs are shown in Table 1. All transgenic pigs harbouring the SV 40- and the MT-Mx gene construct had extensive rearrangements (Müller et al., 1992b). The efficiency of gene transfer with the SV-Mx construct decreased dramatically. Using the mMx-Mx construct all eight transgenic pigs were shown by Southern blotting, PCR, and differential restriction analysis to have correct integration of 10–30 copies and stable transmission to offspring. The IFN-inducibility of the transgenes had been documented in vitro and in vivo. In two of five transgenic lines an IFN-inducible increase of transgene mRNA levels was found. We were not able to detect an increase of murine Mx1 protein in the examined cells and tissues of the transgenic pigs (Müller et al., 1992b). Probably the response of the transgenes to IFN is too low to result in detectable amounts of protein. Arnheiter et al. (1990) have found that Mx transgenic mice may be non-expressing or, alternatively, they may be low- and high-responder animals. High responders were protected against virus infection, while low responders are not or only when infected with high virus doses.

ANTIBODY GENES

As shown by many investigations, cloned genes of monoclonal antibodies (mAb) can be expressed in large amounts in transgenic mice after the transfer of suitable gene constructs into the germ line. These mice produce antibodies against specific antigens without any prior immunisation or contact. In an attempt to study the expression pattern of immunoglobulin-encoding genes in transgenic livestock, we have introduced the genes for the light and heavy chain of a mouse monoclonal antibody into the germ-line of mice (control), rabbits, and pigs (Weidle et al., 1991). The experiments were designed to evaluate whether antibodies could be produced in large amounts in the serum of those animals. Furthermore, transgene antibody expression would be a first step in a so-called "genetic immunisation" strategy against bacterial and viral diseases. We have used the Ig heavy and light chain genes (encoding a 9.25 kb λ and 5.5 kb κ chain), from an anti-idiotypic antibody directed against mAb-NP, which is directed against the hapten 4 hydroxy-3-nitrophenylacetate (NP) in two different gene configurations for gene transfer. Integration was detected in three founder mice (1–3 copies) and three rabbits (40–60 copies) and two pigs (~50 copies). The serum levels of reconstituted antibodies of living founder ani-

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TABLE 1. Comparison of Success Rates With Different Mx Gene Constructs

Gene construct	$MTII_A$Mx	SV40-Mx	MxMx
Number of transfers (n)	22	19	40
Embryos transferred (n)	1,083	809	1,629
Pregnancy rate (%)	41	37	40
Piglets born (n)	22	26	77
Survival rate			
Total (%)	2	3	5
In pregnant recipients (%)	5	9	11
Transgenic piglets (n)	6	1	8
Integration rate (%)	27	4	10
Total efficiency (%)	0.6	0.1	0.5

mals were quantified by ELISA. This assay gives a measure of the specific antigen binding activity (no cross-reactivity with control sera was observed) but does not yield information about the exact composition of the antibody. Titers of 100–300 μg mAb/ml in transgenic rabbits and up to 1,000 μg mAb/ml in one transgenic pig were measured in the sera of the founder animals. The transgene was transmitted to the offspring of transgenic mice, rabbits, and the pig and similar serum levels of antibody were detected in transgenic progeny.

In two transgenic mouse lines 20 and 38 μg/ml were detected and a markedly increased level up to 150 μg in the offspring of one line (presumably the founder mouse was a mosaic). The total serum Ig titers were about 2.5 mg/ml serum in all three species (Weidle et al., 1991).

The characterisation of the composition of the antibodies expressed in transgenic rabbit and pig sera was investigated by isoelectric focusing and subsequent immunofixation by incubation with anti Fcγ serum and silver staining of the complexes. Surprisingly only a minor portion of antiserum from the transgenic mice was found to contain antibodies coinciding with the purified antibody from the ascites fluid whereas serum of transgenic rabbits did not show any discrete bands. We conclude that the smear present in crude serum might be indicative for heterologous antibody by association of the light chains of the rabbit and pig with the heavy chain of the mouse. The level of mouse κ-chain expression might be insufficient for complete allelic exclusion (Weidle et al., 1991). An association of heterologous chains with those of the mouse seems not to be probable. Further investigations are necessary for clarifying these problems.

MAMMARY SPECIFIC PROMOTERS

The ability to express genes with tissue-specific promoters in the mammary glands is essential for the secretion of foreign proteins into the milk of transgenic animals (i.e., gene farming). To direct the expression of the hGH (human growth hormone) to mammary glands of mice, rabbits, and pigs, genomic hGH coding sequences have been linked to murine WAP (whey acidic promoter) sequences. We have produced 32 transgenic mice, 10 transgenic rabbits, and 4 transgenic pigs which had integrated either 110 bp (WAP1) or 2.4 kb (WAP 2) of the 5′ flanking region (Brem et al., 1991). Expression was investigated by RNA-slot-blot, immunohistochemistry, Northern blotting, and in situ hybridisation in tissues and by RIA in serum of milk and blood of transgenic animals. Expression levels in milk serum were rather low in mice at around 6–200 ng/ml, in rabbits up to 4,000 ng/ml, and in pigs between 10 and 40 ng/ml (unpublished data). RNA analyses indicated that a higher amount of protein is expected because in some mice RNA levels of the foreign hGH gene approaches the levels of the mRNA of the endogenous WAP gene (Günzburg et al., 1991). On the one hand this suggests that there might be problems with the translation of the transgene mRNA of hGH in the mammary gland; on the other hand we are not quite sure whether we have detected all hGH-protein in the milk serum because of using a monoclonal antibody which only detects monomeric hGH but possibly not dimeric or trimeric forms. We found no significant increase of GH in the serum of WAP-hGH transgenic mice, rabbits, or pigs. All animals looked healthy and were fertile, and no changes in growth parameters were observed. However, when other organs from transgenic mice were examined, high-level expression of hGH was unexpectedly observed in the brains of mice. This expression was seen to occur specifically in Bergmann glia cells (Günzburg et al., 1991; Brem et al., 1991). By comparison with other gene constructs we propose that the combination of the WAP promoter and the hGH structural gene results in a novel tissue specificity in the Bergmann glia.

In order to achieve high yields of foreign proteins in the milk we have chosen in further experiments the regulatory sequences of the bovine α_{S1}-casein gene and constructed several fusion genes with different structural genes. We have generated 14 lines of transgenic rabbits carrying three different modifications of the α_{S1} gene regulatory sequences linked to bovine prochymosin gene. The chymosin (rennin) gene codes for an inactive 40 kD prorennin, which is normally secreted and activated in the stomach of calves. We expected the inactive prorennin to be secreted. After purification the prorennin is autocatalytically activated in vitro at a pH between 2 and 3 or 4.5 which results is a 38 kD prorennin or 36 kD pseudoprorennin, both of which are equally active. Yields were found in the milk of transgenic rabbits with respect to the functional prorennin to be as high as 10 g/L in at least two founders with different constructs. There were no obvious differences between the three different modifications (Brem and Hartl, 1991) of the α_{S1} casein promoter, but up to now not all of the transgenic lines have been tested for expression. We have modified our system in a way that we were also able to insert and express cDNA's. This transcription cassette has already been used in transgenic rabbits and these animals show a secretion level between 0.5 and 2 g/l.

Using a milking machine we were able to collect more than 200 ml milk from a rabbit every second day.

Thus it should be possible to get 10 kg milk per rabbit and year and to produce in this amount of milk up to 100 g foreign protein per rabbit and year.

Because our bovine gene constructs function correctly in rabbits, we expect that they should also work comparably in transgenic cattle.

CONCLUSIONS

Gene transfer in livestock is a very promising tool not only for changing growth parameters but also especially for high level protein production in body fluids. This gene farming procedure opens the possibility to collecting proteins with the necessary posttranslational modifications for diagnostic, therapeutic, or nutritive purposes in high amounts and at relative low prices.

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